Substitutions at residues 300 and 389 of the VP2 capsid protein serve as the minimal determinant of attenuation for canine parvovirus vaccine strain 9985-46

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Abstract
Identifying molecular determinants of virulence attenuation in live attenuated canine parvovirus (CPV) vaccines is important for assuring their safety. To this end, we identified mutations in the attenuated CPV 9985-46 vaccine strain that arose during serial passage in Crandell–Rees feline kidney cells by comparison with the wild-type counterpart, as well as minimal determinants of the loss of virulence. Four amino acid substitutions (N93K, G300V, T389N and V562L) in VP2 of strain 9985-46 significantly restricted infection in canine A72 cells. Using an infectious molecular clone system, we constructed isogenic CPVs of the parental virulent 9985 strain carrying single or double mutations. We observed that only a single amino acid substitution in VP2, G300V or T389N, attenuated the virulent parental virus. Combinations of these mutations further attenuated CPV to a level comparable to that of 9985-46. Strains with G300V/T389N substitutions did not induce clinical symptoms in experimentally infected pups, and their ability to infect canine cells was highly restricted. We found that another G300V/V562L double mutation decreased affinity of the virus for canine cells, although its pathogenicity to dogs was maintained. These results indicate that mutation of residue 300, which plays a critical role in host tropism, is not sufficient for viral attenuation in vivo, and that attenuation of 9985-46 strain is defined by at least two mutations in residues 300 and 389 of the VP2 capsid protein. This finding is relevant for quality control of the vaccine and provides insight into the rational design of second-generation live attenuated vaccine candidates.

INTRODUCTION
Canine parvovirus (CPV) causes a severe, highly contagious gastroenteric disease in pups. Dogs become infected with CPV through the oronasal route. Following an incubation period, which ranges from 4 to 6 days, animals develop acute enteritis, clinically characterized by anorexia, vomiting, pyrexia, diarrhea (e.g. mucoid and hemorrhagic diarrhea) and leukopenia [1].

CPV, which belongs to the genus Protoparvovirus in the family Parvoviridae, is an autonomous, non-enveloped virus that contains a linear, negative-sense, single-stranded DNA genome of about 5 kb [2]. The genome contains 3’ and 5’ palindromic sequences in the non-coding hairpin region and two major ORFs that encode non-structural proteins (NS: NS1 and NS2) and capsid proteins (VP: VP1, VP2 and VP3) [3]. The amino-terminal ends of NS1 and NS2 overlap and are identical in sequence. The carboxy-terminal end of the NS2 protein is derived from differential splicing of mRNA, and the protein is translated in an overlapping but different reading frame from that of NS1. VP1 and VP2 are translated from alternatively spliced mRNAs, with the VP2 sequence completely contained within that of VP1, which has an additional amino-terminal sequence [4]. VP3 is processed by cleavage of 15 to 20 amino acids from the amino terminus of VP2 [5, 6]. VP2 is the major component of the parvovirus capsid, and amino acid substitutions in this protein cause changes in the antigenic properties and cell tropism of CPV, as well as in the pathogenesis of parvoviruses [5, 7–10].

CPV-2 was first identified in 1978 from outbreaks in dogs. Over the subsequent four decades, new antigenic variants have evolved. CPV-2 is closely related to feline parvovirus (FPV); therefore, it is assumed to be a host variant of FPV.
In the 1980s, the original CPV-2 type was completely replaced by two new antigenic variants in dogs, which were termed CPV types 2a and 2b. In 2000, CPV-2c, carrying an D426E substitution, was reported in Italy [11, 12]. The canine host range of the emerging virus was defined by a combination of mutations in the VP2 gene that corresponded to three regions on the surface of the capsid protein. These mutations were identified as K93N, D323N and either G299E or A300D [9].

Focusing on these CPV variants, live attenuated vaccines were developed and have been used in many countries for the last 30 years [13, 14], with new type 2b vaccines having been developed recently [15]. Serial passage of wild-type strains of CPV in cell culture is the method most commonly applied to generate the attenuated vaccine strains currently in use [14, 16]. However, the criteria for selecting attenuated strains are highly empirical, and little is known about the molecular mechanisms underlying attenuation. Therefore, determinants of attenuation must be understood to control the quality of these vaccines.

Although reversion of virulence in a live attenuated CPV vaccine has never been demonstrated and the attenuation of virulence has been proven to be highly stable [13], concerns have arisen that enteric illnesses observed in animals following administration of a CPV vaccine may have resulted from vaccine reversion to a virulent form [17]. It is important to clearly discriminate attenuated vaccine strains from wild-type strains to resolve this concern. Therefore, it is necessary to determine the genetic markers that define the attenuated phenotype.

We developed the CPV 9985-46 live attenuated vaccine strain in 2015 by serially passaging a wild-type isolate in Crandell–Rees feline kidney (CRFK) cells. Its protective efficacy was demonstrated in a challenge test conducted in our laboratory (Table 1). The efficacy and safety of the vaccine has been certified in a clinical trial under Japanese good clinical practice (GCP) guidelines in pre-licensure status. To determine the genetic basis for the attenuation of the CPV 9985-46 vaccine strain, we identified mutations that arose in the attenuated CPV during serial passage in cell culture by comparison with its wild-type counterpart, as well as minimal determinants of the loss of virulence through generation of molecularly cloned viruses carrying mutations introduced by site-directed mutagenesis to an infectious clone of the wild type. We identified the minimal combination of amino acids for the attenuation.

### RESULTS

#### Protective efficacy of 9985-46 vaccine strain

The challenge inoculation of dogs with virulent CPV type 2b was performed after immunization with the 9985-46 vaccine strain. As summarized in Table 1, the dogs of the 9985-46-immunized group did not exhibit any clinical symptoms of the disease, while the dogs of the non-immunized control group developed clinical symptoms that are typically related to CPV infection, and shed virus from the rectum. Additionally, the immunized animals with a neutralizing antibody titre greater than 1:640 after the challenge did not shed any virus. Based on these findings, it was concluded that the 9985-46 vaccine strain conferred protective immunity against CPV infections virulent to vaccinated animals.

#### Virulence of the vaccine strain and its parental strain

We examined the virulence of the 9985-46 vaccine strain and the parental 9985 strain in pups by exposing four animals each through natural infection per os for the better modelling of disease development than by injection. Strain 9985 induced typical signs of CPV infection, such as diarrhoea, leukopenia, pyrexia, anorexia, depression and dehydration, in four pups. One pup became moribund at 8 days post-inoculation (p.i.). In contrast, none of the pups inoculated with strain 9985-46 developed any signs of disease (Table 2, groups A and B). Based on these results, we concluded that the 9985-46 vaccine strain acquired an attenuated phenotype, as compared to its virulent parental strain, through serial passage in feline cells.

#### Identification of mutations in the vaccine strain genome

Whole genomes of vaccine strain 9985-46 and the parental strain 9985 were sequenced, except for the non-coding regions at the 5’ and 3’ termini. The sequence of 9985-46

### Table 1. Protective efficacy of canine parvovirus (CPV) vaccine strain 9985-46 determined by challenge test in dogs

<table>
<thead>
<tr>
<th>Immunization</th>
<th>Dog no.</th>
<th>Serum neutralizing antibody titre at day of challenge</th>
<th>Clinical signs after inoculation with virulent CPV†</th>
<th>Viral shedding from rectum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Diarrhoea</td>
<td>Leukopenia</td>
</tr>
<tr>
<td>9985-46 vaccine strain</td>
<td>1</td>
<td>1:3620</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1:640</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1:80</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>None</td>
<td>4</td>
<td>&lt;1:10</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>&lt;1:10</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

†Virulent CPV type 2b 9690 strain.

†Hyperthermia, loss of appetite, depression and dehydration.
differed by seven nucleotides, of which one was caused by a silent mutation, from that of 9985 resulting in two amino acid substitutions in the NS1 protein (I574M and D665G) and four in the VP2 capsid protein (N93K, G300V, T389N and V562L; Table 3). All of these residues in VP2 were expressed on the surface of the viral capsid [18]. One of the axes of symmetry in the icosahedral CPV particle, known as the 3-fold spike, contains residues involved in binding to the transferrin receptor (TfR) and infection, and this is the central domain determining the host range of CPV [19]. VP2 residue 300 is located in the shoulder region of the 3-fold spike of the capsid and determines canine specificity by TfR binding [20]. Residue 389 is located in the vicinity of residue 300 on the capsid surface [21]. Mutations of residues 93 and 562 have been suggested to be important for adaptation of CPV to other carnivore cell lines [7].

Assembly of full-length DNA clones and the rescue of modified CPVs from these DNA clones

Genomic analysis revealed that vaccine strain 9985-46 accumulated amino acid substitutions in both the NS and VP genes. To construct molecular clones 9985 and 9985-46, we ligated DNA fragments containing the coding sequences of the NS and VP genes, which were prepared from each viral isolate, with the 3¢ and 5¢ terminal non-coding regions synthesized based on published sequences. Although there could be nucleotide differences in the non-coding regions of these viruses, it is conceivable that they do not play critical roles in the attenuation of 9985-46 because the molecularly constructed viruses possessed biological properties similar to their original viral counterparts both in vitro and in vivo. We are confident that mutation(s) in the NS and VP genes, but not in the 3¢ and 5¢ terminal regions, define the attenuation of virulence in vaccine strain 9985-46. We constructed isogenic molecular clones of the virus by introducing the entire NS or VP gene from the attenuated clone into the backbone of a pathogenic counterpart, rescued them in cell culture and assessed their pathogenicity in pups. While the virus (v9985-VP) carrying the VP gene of the attenuated clone did not cause clinical manifestations in pups, the one (v9985-NS) carrying the NS gene of the attenuated clone induced pyrexia, loss of vigorous prostration and weight loss.

<table>
<thead>
<tr>
<th>Group</th>
<th>Virus</th>
<th>Inoculum*</th>
<th>Diarrhoea</th>
<th>Leukopenia</th>
<th>Presence of signs†</th>
<th>Mortality</th>
<th>Days post-infection euthanasia/necropsy</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>9985 parental strain</td>
<td>10^8.5</td>
<td>4/44</td>
<td>2/4</td>
<td>4/4</td>
<td>1/4</td>
<td>8, 14, 14, 14</td>
</tr>
<tr>
<td>B</td>
<td>9985-46 vaccine strain</td>
<td>10^8.5</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>0/4</td>
<td>14, 14, 14, 14</td>
</tr>
<tr>
<td>C</td>
<td>v9985-NS</td>
<td>10^8.0</td>
<td>0/3</td>
<td>0/3</td>
<td>2/3</td>
<td>0/3</td>
<td>14, 14, 14</td>
</tr>
<tr>
<td>D</td>
<td>v9985-VP</td>
<td>10^8.0</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>0/2</td>
<td>14, 14</td>
</tr>
<tr>
<td>E</td>
<td>v9985</td>
<td>10^8.5</td>
<td>4/4</td>
<td>4/4</td>
<td>4/4</td>
<td>2/4</td>
<td>7, 7, 14, 14</td>
</tr>
<tr>
<td>F</td>
<td>v9985-46</td>
<td>10^8.5</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>7, 11, 14</td>
</tr>
<tr>
<td>G</td>
<td>v9985-G300V</td>
<td>10^0.5</td>
<td>1/4</td>
<td>0/4</td>
<td>1/4</td>
<td>0/4</td>
<td>7, 11, 14, 14</td>
</tr>
<tr>
<td>H</td>
<td>v9985-T389N</td>
<td>10^8.5</td>
<td>0/3</td>
<td>0/3</td>
<td>2/3</td>
<td>0/3</td>
<td>7, 14, 14</td>
</tr>
<tr>
<td>I</td>
<td>v9985-V562L</td>
<td>10^6.5</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
<td>6, 6, 6</td>
</tr>
<tr>
<td>J</td>
<td>v9985-N93K/G300V</td>
<td>10^6.5</td>
<td>1/3</td>
<td>0/3</td>
<td>3/3</td>
<td>0/3</td>
<td>7, 14, 14</td>
</tr>
<tr>
<td>K</td>
<td>v9985-N93K/V562L</td>
<td>10^8.0</td>
<td>3/3</td>
<td>1/3</td>
<td>3/3</td>
<td>0/3</td>
<td>7, 14, 14</td>
</tr>
<tr>
<td>L</td>
<td>v9985-G300V/T389N</td>
<td>10^6.5</td>
<td>0/7</td>
<td>0/7</td>
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</tr>
<tr>
<td>M</td>
<td>v9985-G300V/V562L</td>
<td>10^0.5</td>
<td>3/3</td>
<td>2/3</td>
<td>3/3</td>
<td>0/3</td>
<td>7, 14, 14</td>
</tr>
<tr>
<td>N</td>
<td>v9985-T389N/V562L</td>
<td>10^6.5</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>7, 14, 14</td>
</tr>
</tbody>
</table>

*TCID<sub>50</sub> ml<sup>−1</sup> x 5 ml.
†Hyperthermia, loss of appetite, depression and dehydration.
‡Positive dogs/tested dogs.
loss (Table 2, groups C and D). Based on these findings, we hypothesized that VP might play an important role in the observed attenuation of CPV 9985-46 and initiated investigations to identify the minimal determinant in the VP gene responsible for virus attenuation.

In order to determine the mutations that were responsible for loss of virulence, either single or double mutations that resulted in N93K, G300V, T389N and V562L substitutions in VP2 were introduced into an infectious clone that was derived from the virulent 9985 strain (Fig. 1), and then, modified viruses were rescued through the transfection of CRFK cells with the DNA clones. The rescued viruses were designated as v9985, v9985-N93K, v9985-G300V, v9985-T389N, v9985-V562L, v9985-N93K/G300V, v9985-N93K/V562L, v9985-G300V/T389N, v9985-G300V/V562L, v9985-T389N/V562L and v9985-46. Transfection with p9985-N93K/T389N did not produce viable virus.

As shown in Fig. 2, these rescued viruses were found to be infectious, exhibiting titres ranging from $10^{6.5}$ to $10^{8.19}$ TCID$_{50}$ ml$^{-1}$ in CRFK cells, except for v9985-N93K and v9985-N93K/V562L. The infectious titre of v9985-N93K was extremely low, $10^{2.88}$ TCID$_{50}$ ml$^{-1}$, and that of v9985-N93K/V562L was relatively low, $10^{5.00}$ TCID$_{50}$ ml$^{-1}$, in CRFK cells. Sequencing of the NS and VP genes of the rescued virus stocks confirmed that these sequences were identical to those of the DNA clones.

**Cell tropism in vitro**

An analysis of the infectious titres of the 9985 parent strain and 9985-46 vaccine strain in feline CRFK and canine A72 cells revealed a salient difference. The 9985 titre was $10^{6.5}$ TCID$_{50}$ ml$^{-1}$ in CRFK cells and $10^{3.5}$ TCID$_{50}$ ml$^{-1}$ in A72 cells, whereas those of 9985-46 were $10^{8.19}$ TCID$_{50}$ and $10^{7.5}$ TCID$_{50}$ ml$^{-1}$, respectively. These results indicate that 9985-46 was replication competent in CRFK cells, but had a
replication defect in A72 cells through serial passage in CRFK cells (Fig. 2). It is possible that serial passage altered the host range of the virus.

The mutants that were replication competent in CRFK cells, along with v9985 and v9985-46, were next investigated for their comparative infectiousness in feline CRFK and canine A72 cells, to gain an understanding of the relationship between the change in host range and viral attenuation. As shown in Fig. 2, the relative abilities of modified CPVs to infect feline CRFK and canine A72 cells revealed three levels of replicative restriction in A72 cell lines: (i) modified CPVs that were severely restricted (≥10^4-fold reduction in titre) in their ability to replicate in A72 cells; v9985-46, v9985-VP, v9985-N93K/G300V, v9985-G300V/T389N and v9985-G300V/V562L, similar to that of the 9985-46 vaccine strain; (ii) modified CPVs that were restricted in their ability to infect A72 cells (2- to 4-log_10-lower titre than that seen in CRFK cells), namely v9985-G300V, v9985-N93K/G300V, and v9985-T389N/V562L; (iii) modified CPVs that were not restricted (<10^2-fold) in their ability to infect A72 cells; and v9985, v9985-NS, v9985-T389N and v9985-V562L, which showed replication similar to that of the original virulent strain 9985. Taken together, a mutation at G300V is sufficient to restrict the ability of the modified CPVs to infect canine cells.

**Virulence in vivo**

To identify the minimum determinants of the VP2 residue that contribute to the observed attenuation of CPV 9985-46, additional pups were experimentally infected. The parental and modified viruses (v9985, v9985-46, v9985-G300V, v9985-T389N, v9985-V562L, v9985-N93K/G300V, v9985-N93K/V562L, v9985-G300V/T389N, v9985-G300V/V562L and v9985-T389N/V562L) productively replicated in all pups, as evidenced by faecal virus shedding, development of viraemia and induction of antiviral neutralizing antibodies. Table 2 summarizes the clinical observations in dogs.
infected with the parental and modified viruses (groups E–N). Experimental infection of pups with the modified CPVs revealed four grades of clinical manifestation: (i) bloody diarrhoea, severe leukopenia and mortality, induced by v9985 and v9985-V562L; (ii) watery diarrhoea but no mortality, caused by v9985-N93K/V562L and v9985-G300V/V562L; (iii) mild symptoms such as mucoid diarrhoea in response to infection by v9985-G300V, v9985-T389N, v9985-N93K/G300V and v9985-T389N/V562L; and (iv) no symptoms observed following infection with v9985-46 and v9985-G300V/T389N.

Fig. 3(a) shows the sum of the recorded clinical scores from 0 to 6 days p.i., during which time all dogs in all groups were alive. The scores of groups inoculated with v9985-46, v9985-G300V, v9985-T389N, v9985-N93K/V562L, v9985-N93K/G300V and v9985-N93K/T389N/V562L or v9985-G300V/T389N/V562L were significantly lower than that of the group infected with v9985 (P<0.05). Among the modified viruses carrying single or double mutations, v9985-G300V/T389N was the only strain that did not result in clinical manifestations other than the rescued attenuated v9985-46.

**Histopathological examination of intestines**

To further investigate the degree of intestinal disease, we performed histopathological examinations on the intestines of pups inoculated with the modified CPVs. Fig. 3(b) shows the histopathological scores for the intestines of dogs inoculated with modified CPVs. The scores for dogs inoculated with v9985-46, v9985-G300V, v9985-T389N, v9985-N93K/G300V, v9985-N93K/V562L, v9985-G300V/T389N or v9985-T389N/V562L were markedly lower than those for dogs inoculated with v9985, v9985-V562L or v9985-G300V/V562L. Severe necrosis of epithelial cells, crypt degeneration in the ileum and jejunum and lymphocyte necrosis in the colon (Fig. S1, available in the online Supplementary Material).

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**Fig. 3.** (a) Average of the sum of clinical scores measured between 0 and 6 days p.i. of dogs inoculated with the modified CPVs. The error bar represents the standard error of the mean from the sum of scores in individual dogs. (b) Intestinal histopathological scores in pups inoculated with modified CPVs. The graph for the v9985-inoculated group shows the average scores of two dogs with mortality at 7 days p.i. The graph for the v9985-V562L-inoculated group shows the average scores of three dogs with mortality at 6 days p.i. The graph for the other groups show the score for one dog euthanized at 7 days p.i. The error bar represents the standard error of mean of the scores observed in individual dogs.
Material) were observed in two v9985-infected dogs that died at 7 days p.i. and in three v9985-V562L-infected dogs that died at 6 days p.i. Severe to moderate necrosis of epithelial cells, crypt degeneration in the ileum and jejunum and mild lymphocyte necrosis in the colon were detected in one v9985-G300V/V562L-infected dog that survived and was subjected to necropsy at the end of the observation period at 7 days p.i. Dogs inoculated with v9985-46, v9985-G300V, v9985-T389N, v9985-N93K/G300V, v9985-N93K/V562L, v9985-G300V/T389N or v9985-T389N/V562L developed only mild lesions. These results indicate that the severity of intestinal lesions was consistent with that of observed clinical manifestations in infected dogs.

Replication in vivo

To obtain additional information on viral dynamics, we investigated viral shedding loads from the rectums of experimentally infected pups. Fig. 4 shows a comparison of the infectious titres of the modified CPVs in rectal swabs. Rectal shedding of virus was detected in all dogs and peaked at 4 or 6 days p.i. Peak levels of viral shedding in dogs infected with the rescued attenuated v9985-46 virus ($10^{5.83}$ TCID$_{50}$ ml$^{-1}$ at 6 days p.i.) were significantly lower than those in dogs infected with the rescued virulent v9985 virus ($10^{8.75}$ TCID$_{50}$ ml$^{-1}$ on 4 days p.i., $P<0.05$). Infection with v9985-T389N or v9985-G300V/T389N, which resulted in mild or no clinical signs and only mild intestinal lesions in pups, also resulted in significantly lower peak titres of shed virus than that induced by v9985 ($10^{6.67}$ TCID$_{50}$ ml$^{-1}$ on 4 days p.i. and $10^{6.07}$ TCID$_{50}$ ml$^{-1}$ on 6 days p.i., respectively; $P<0.05$). These lower viral shedding levels were consistent with the viral attenuation observed in pups.

Serological response

In order to evaluate the immunogenicity of the rescued viruses, sera from the dogs that were infected with these viruses were subjected to a neutralization test with the strain 9985 as the target virus. After 14 days p.i., the neutralizing antibody titres of the animals that were infected with v9985-G300V/T389N were comparable to those that were infected with the rescued attenuated v9985-46 strain. The antibody titres were approximately four times lower than those of the animals that were infected with the rescued virulent v9985 strain (Fig. S2).

Confirmation of genetic stability of the modified virus

PCR and sequencing were performed to examine the genomic stability of modified viruses in rectal swabs. The sequence analysis showed that the modified viruses replicated with high fidelity in the pups, and that their genomic sequences did not revert to those of the parental strains.

DISCUSSION

The live attenuated CPV 9985-46 vaccine is safe and effective; however, the genetic determinants of attenuation have not yet been defined. The current study was designed to elucidate the minimal genetic determinants required for the attenuation of CPV 9985-46 virulence in dogs.

Fig. 4. Viral shedding kinetics in pups inoculated with modified CPVs. Average titres and standard errors (error bars) in each group are shown. Rectal samples were collected from all pups until 6 days p.i. After 6 days p.i., sample numbers in each group are reduced because of euthanized pups for necropsy by the collection of the blood samples on 14 days p.i., as described in Table 2.
In the current study, we observed that only a single amino acid substitution in VP2, G300V or T389N was sufficient to cause attenuation of a virulent parental virus. A single mutation at position 300 is known to significantly alter receptor binding and infectivity in various carnivore species [20]. Our findings indicate that a single mutation resulting in G300V attenuates CPV, reinforcing the idea that the amino acid at that position in VP2 is a key residue that determines the host cell tropism and pathogenicity of CPV. In addition, we found that the T389N amino acid substitution is also responsible for attenuation. There are scant reports of changes at this position governing biological properties of CPV. One such study, however, speculates that a structural change in CPV caused by a single amino acid substitution at this position may result in non-viable virus because of the restriction of host range [21]. In that study, the authors did not examine the biological properties of the mutant. In this study, we showed for the first time that a mutation at position 389 reduced CPV pathogenicity.

Combined G300V and T389N substitutions in VP2 further attenuated CPV. Titres of shed virus indicated that this combination of mutations could reduce viral replication in the intestines of pups. CPV replication has been shown to occur initially in oropharyngeal lymphoid tissue and subsequently in the intestines through hematogenous spread [22]. Although CPV has a broad tissue tropism [23], it replicates in the dividing cells in the crypts of the intestine because of its preference for rapidly dividing cells. Therefore, the degree of viral shedding from the rectum is a legitimate indicator of viral replication in vivo. Furthermore, the severity of intestinal lesions determines the severity of clinical disease [22]. For these reasons, the combined substitutions of G300V and T389N in VP2 resulted in reduced viral replication and tissue damage in the intestines, indicating viral attenuation in pups.

G300V, in collaboration with T389N, altered the host range of the virus. The CPV capsid forms a core eight-stranded β-barrel motif, with some β-strands connected by extensive loop structures that form the capsid surface [24, 25]. Three molecules of VP2 interact at the shoulder region of the 3-fold spike of the viral particle. Residue 300 of VP2 is located on the exposed loop of one of the monomers in the shoulder region that facilitates binding to TfR in domestic dogs [8, 18, 20, 21, 26]. Residue 389 is located on the exposed loop of another monomer in the shoulder region. T389 forms a hydrogen bond with E298 in the loop where residue 300 resides [21, 26]. Therefore, it is possible that both G300V and T389N cause structural changes in the shoulder region that binds TfR in collaboration with the two residues. Taken together, it is conceivable that simultaneous G300V and T389N substitutions are important for increasing affinity of the virus for an alternative host.

However, decreased affinity to the original host cells is not in itself sufficient to explain viral attenuation. This notion is supported by the mutant virus carrying G300V/V562L to canine cells, which showed reduced affinity but maintained pathogenicity to the host. VP2 residue 562 appears important for host adaptation because the residue lies adjacent to residues 564 and 568, which are important for efficient viral replication in cats [7, 26, 27]. This finding suggests that improved binding to the receptor of alternative host cells may be distinct from attenuation of virulence in the original host. Also, there is an example of CPV attenuation through serial passages in cells of canids, which are the original host species of the virus. The attenuated CPV strain, which was derived by Badgett et al. [16] after 80 serial passages in canine cells, contained amino acid substitutions neither at residue 300 in VP2 nor at the residues in close proximity, which are known to be the key residues for the alteration of tropism. The findings suggest that virus attenuation could occur by other mechanism(s) without the alteration of host specificity. In infectious bursal disease virus (IBDV), mutations in the major capsid protein of the virus are sufficient to confer cell tropism and replication efficiency, but do not necessarily lead to attenuation of the virus [28]. Other than interactions of the capsid with TfR, there may be several other mechanisms for viral attenuation that are mediated by changes in the protein. The VP2 protein may interact with not only the cellular receptor but also other cellular proteins. In IBDV, the viral structural protein interacts with host cellular ribosomal proteins or transmembrane proteins located in the Golgi apparatus [29, 30]. It is also possible that amino acid changes in VP2 have greater effects on nuclear localization of the viral genome than on receptor binding. The minute virus of mice, for example, has functional nuclear localization signals in its capsid proteins [31]. Taken together, these findings suggest that mechanisms of virulence attenuation involve more than just alterations that affect affinity to the receptor.

We hypothesized that altered affinity was linked to viral attenuation. Contrary to our assumption, G300V/T389N and G300V/V562L, which exhibited lowered infectivity in canine cells and markedly different disease phenotypes in vivo, support the idea that a mutation at residue 300 is not sufficient for viral attenuation in vivo, but that it plays a critical role in host tropism. This study clearly shows that attenuation of the CPV 9985-46 vaccine strain is defined by additional substitutions of key amino acids that interact with the altered residue 300. Here, we show that amino acid substitutions G300V/T389N not only alter affinity of the virus for host cells but, together, are also the definitive genetic marker of the attenuated phenotype of vaccine strain 9985-46. Although not all attenuated CPV vaccine strains carry amino acid substitutions at residues 300 and 389, as did the variant that was described by Badgett et al., our results show that a CPV strain carrying substitutions at least at residues 300 and 389 could be attenuated in the context of the 9985-46 strain. Further studies would reveal whether these substituted residues serve as the key residues for other CPV strains.

In this study, we did not conduct a challenge test because we experimentally infected animals with mutant viruses via
the natural route of infection in order to examine their pathogenicity. Serological analyses revealed that the neutralizing antibody titre of the dogs that were infected by the double mutant at residues 300 and 389 was comparable to that of the dogs that were infected by the attenuated 9985-46 strain, which confirmed that its immunogenicity was adequate for use as a vaccine. A challenge test to prove its protective efficacy would be required in the future.

In conclusion, this study indicates that attenuation of CPV strain 9985-46 is mediated by two mutations in the VP2 capsid protein at residues 300 and 389. The presence of both mutations is necessary and sufficient to obtain the attenuated phenotype. These results are important for quality control of the 9985-46 live attenuated vaccine strain and provide insight into the rational design of second-generation live attenuated vaccine candidates.

METHODS

Cells and viruses

CRFK cells and A72 canine tumour cells were obtained from ATCC. CRFK cells were cultured in minimum essential medium (MEM) with 5 % FBS at 37 °C. A72 cells were initially cultured in Leibovitz’s L15 medium with 5 % FBS and then adapted to grow in MEM with 5 % FBS.

The attenuated CPV strain 9985-46 examined in this study was developed as a pre-licensure vaccine strain. The efficacy and safety of the vaccine strain were established in 2015 in a clinical trial with dogs under Japanese GCP guidelines. The parent CPV type 2b 9985 strain was isolated from the tonsil tissue of a dog that died from CPV infection. CPV 9985 was subjected to passage and cloned five times by the limiting dilution method in CRFK cells at 37 °C. The virus was subjected to serial passages in CRFK cells at 32 °C. The infective titre reached approximately 1 × 10⁸ TCID₅₀ ml⁻¹ at the end of each passage, and the culture was diluted 1:100 to initiate the next passage. The virus was plaque-purified in CRFK cells thrice at passage 41 and subjected to an additional two passages in CRFK cells. The resulting virus was designated strain 9985-46. In order to evaluate its protective efficacy, a challenge test with virulent CPV was performed. Two 2-month-old and one 1-month-old puppies from a specific pathogen-free CPV-negative dog farm at Kyoto Biken Laboratories, Inc. were studied. Two dogs were immunized with an injection of 10⁵ TCID₅₀ of the CPV strain 9985-46 15 days before the challenge, and one dog was immunized with an injection of 10⁷ TCID₅₀ of the 9985-46 strain 50 days before the challenge. All animals, in addition to two control dogs (2 months old), were challenged orally with 5 ml containing 10⁵ TCID₅₀ of CPV strain 9690 of virulent CPV type 2b. The clinical reactions of the dogs were evaluated by daily observation and recording of body temperature and weight gain, and bi-weekly monitoring of the white blood cell count after the challenge. Rectal swabs for viral shedding tests were collected on days 2, 4, 6, 8, 10 and 12 after the challenge. Virus shedding was assessed by conducting the virus titration of rectal swabs.

For the sequence analysis, viral DNAs were extracted directly from the virus stocks of the 9985-46 vaccine strain and the parental 9985 strain using a QIAamp DNA Mini Kit (Qiagen). Two overlapping PCR products (between bases 241 and 3174 and between bases 2744 and 4585) were amplified with the KOD DNA polymerase (KOD-Plus-Neo, Toyobo) and then directly sequenced by TaKaRa Bio Dragon Genomics Center, with primers spaced every 400 to 500 bases. Nucleic acid sequencing and a deduced amino acid sequence comparison and alignment were performed using BioEdit version 7.0.9 (Tom Hall, Ibis Biosciences). Mutations at the 3’ and 5’ terminal non-coding sequences were not investigated in this study.

Construction of full-length DNA clones

The synthesized 3’ and 5’ termini of DNA strands from the CPV genome, based on infectious clone pCPV Y1 (GenBank accession no. D26079; nt 1–400 and nt 4607–5075) [32], were obtained from Biomatik. The synthesized DNA, which connected two terminal DNAs (the 3’ and 5’ termini) was cloned into pBMH (Biomatik), a derivative of pBlueScript II SK(+), at multiple modified cloning sites. The plasmid was linearized by inverse PCR using the KOD DNA polymerase and a primer pair (5'–ATTGTACTTGTATTTGATATGAAAAATCTCAACTAGCACAACGTCCAACTAAATGGAAAGGATGTTCGCTGGA–3' and 5'–TTGGACGTTGTCACTTGTTGTGCTCATTTAATATACATTTAATTTAATTAAAATCTT–3') designed to separate the 3’ and 5’ terminal DNAs, such that each terminal sequence was located at an end of the plasmid. CPV genomic DNA, including NS1, NS2, VP1 and VP2 genes, was amplified by PCR with the KOD DNA polymerase and a primer pair (5’–TGTGACAACGTCCAACATAATGGAAAGGATGTTCGCTGGA–3’ and 5’–AAATACGAATCAAATTTTCTATTGCTGTAATTTAATATACATTTAATTTAATTAAAATCTT–3’) designed for insertion by In-Fusion cloning (Clontech Laboratories, Inc.) of the genomic DNA from strains 9985 and 9985-46. The amplified fragments were ligated to the linearized plasmid using an In-Fusion HD Cloning Kit (Clontech Laboratories, Inc.). Escherichia coli DH5α cells (Takara Bio Inc.) were transformed with the ligated plasmids and grown overnight at 37 °C in the presence of 50 µg ml⁻¹ ampicillin. The final assembled, full-length DNA clones were designated p9985 and p9985-46 (DDBJ accession nos. LC270891 and LC270892, respectively). The isogenic clones of p9985-NS and p9985-VP were generated by inserting the entire NS or VP gene of p9985-46 into the backbone of p9985. The genome of p9985 that lacked the VP2 gene was prepared by inverse PCR using KOD DNA polymerase and a primer pair (5’–GTATATGAAAAATCTCAACTAGCACAACGTCCAACTAAATGGAAAGGATGTTCGCTGGA–3’ and 5’–GTGGACGTTGTCACTTGTTGTGCTCATTTAATATACATTTAATTTAATTAAAATCTT–3’), and served as the vector. The VP2 gene of p9985-46 was amplified by PCR with another primer pair (5’–AAGTGAAAAAGACAAATCTCTCTTTTATTACCT–3’ and 5’–TATTGCTTGTGTAATTTAATATACATTTAATTTAATTAAAATCTT–3’) as the insert. The plasmid p9985-VP was constructed by ligating the insert with the vector by In-Fusion cloning. The plasmid p9985-NS was
constructed by replacing the VP2 gene of p9985-46 with that of p9985 using the same strategy. Standard molecular biological techniques were used for cloning [33]. The integrity of these plasmids was confirmed by nucleotide sequencing.

**Site-directed mutagenesis in the VP2 gene**

To introduce mutations into the VP2 gene of p9985 PCR, specific primer pairs were developed. AA93S/AA93AS, AA300S/AA300AS, AA389S/AA389AS and AA562S/AA562AS (Table 4) were synthesized to introduce nucleotide changes at C3062A, G3682T, C3949A and G4467C, respectively. The resulting amino acid substitutions were as follows: N93K, G300V, T389N and V562L in VP2. PCR amplification was performed for 30 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for the primer pairs AA93S/AA93AS, AA300S/AA300AS and AA562S/AA562AS, or 60 °C for the primer pair AA389S/AA389AS for 15 s, and extension at 72 °C for 40 s. The reaction was carried out using PrimeSTAR Max DNA Polymerase (TaKaRa Bio Inc.). E. coli competent cells were transformed with the PCR products to amplify the resulting plasmids, p9985-N93K, p9985-G300V, p9985-T389N and p9985-V562L. To introduce additional site-directed mutations, the above-described procedure was executed with templates already carrying mutations. Standard molecular biology techniques were employed to construct the plasmids [33]. All genomic manipulations were confirmed by nucleotide sequencing.

**Rescue of gene-modified CPVs**

To rescue viruses, CRFK cells were transfected with any one of the following plasmid clones: p9985, p9985-46, p9985-NS, p9985-VP, p9985-N93K, p9985-G300V, p9985-T389N, p9985-V562L, p9985-N93K/G300V, p9985-N93K/T389N, p9985-N93K/V562L, p9985-G300V/T389N, p9985-G300V/V562L and p9985-T389N/V562L, respectively. All of the introduced mutations were confirmed by sequencing.

**Measurement of infectious virus titres in feline CRFK and canine A72 cells**

Infectious units of each virus stock were assessed by end-point titration in CRFK and A72 cell cultures in roller tubes. Each cell suspension was inoculated with 0.1 ml of serial 10-fold dilutions of the virus mixed with 1 ml of MEM and 5 % FBS in a tube. Following static culture for 24 h at 37 °C for adhesion, the culture medium was replaced with 1 ml of MEM with 2 % FBS. The cultures were then incubated on roller drums for 6 days. Virus replication was confirmed by haemagglutination (HA) of pig erythrocytes by the culture supernatant at 4 °C. Those cultures that exhibited HA activity were considered positive for CPV, and the viral titres (TCID50 ml−1) in the respective samples were calculated using the Reed–Müench method [34].

**Virulence studies in dogs**

Forty-nine beagle pups, aged 5–11 weeks and obtained from a specific pathogen-free, CPV-negative dog farm at Kyoto Biken Laboratories, Inc., were assigned to 14 groups and designated groups A–N, comprising 4, 4, 3, 2, 4, 3, 3, 3, 3, 7, 3 and 3 pups, respectively. Each group was placed in a separate room and was further divided into subgroups comprising two or three pups that were group-housed in separate cages. Pups in all groups (A–N) were inoculated orally with a 5 ml suspension of virus strain 9985, 9985-46, v9985, combined with 2.5 μg of each DNA clone followed by incubation for 15 min. A mixture of each clone and FuGENE6 was then added to CRFK cells covered with 5 ml of MEM and 5 % FBS. The transfected cells were incubated at 37 °C. On day 7 post-transfection, the supernatant was collected and the infectious titre was assessed. To propagate the viruses, CRFK cells in 20 ml of MEM containing 5 % FBS in a 75 cm2 cell culture flask were inoculated with the supernatants. Aliquots of the culture supernatant were stored at -80 °C and designated v9985, v9985-46, v9985-NS, v9985-VP, v9985-N93K, v9985-G300V, v9985-T389N, v9985-V562L, v9985-N93K/G300V, v9985-N93K/T389N, v9985-N93K/V562L, v9985-G300V/T389N, v9985-G300V/V562L and v9985-T389N/V562L, respectively. All of the introduced mutations were confirmed by sequencing.

**Table 4. Oligonucleotide primers used in mutagenesis of CPV**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′ to 3′)</th>
<th>Position†</th>
<th>Orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA93S</td>
<td>CAGTTAaGGAAACATGCGTTAGAT</td>
<td>3058–3083</td>
<td>Sense</td>
</tr>
<tr>
<td>AA93AS</td>
<td>TGTTTCTTAACTGCGTCCTATC</td>
<td>3072–3047</td>
<td>Antisense</td>
</tr>
<tr>
<td>AA300S</td>
<td>GAAGGAGTACTAATCTTGGTTATAT</td>
<td>3678–3704</td>
<td>Sense</td>
</tr>
<tr>
<td>AA300AS</td>
<td>GTTGATGCTCCTTCAGCGTGTGGCA</td>
<td>3692–3667</td>
<td>Antisense</td>
</tr>
<tr>
<td>AA389S</td>
<td>AAAACTaACACACAGGAGAAAACC</td>
<td>3940–3970</td>
<td>Sense</td>
</tr>
<tr>
<td>AA389AS</td>
<td>TGGTTGATGTTTTTTGACCAGTTT</td>
<td>3959–3934</td>
<td>Antisense</td>
</tr>
<tr>
<td>AA562S</td>
<td>TAACTATCACCAGGATATTGAGG</td>
<td>4463–4488</td>
<td>Sense</td>
</tr>
<tr>
<td>AA562AS</td>
<td>CTGGGTGATAGTAAATTTGGTATC</td>
<td>4477–4452</td>
<td>Antisense</td>
</tr>
</tbody>
</table>

*The introduced mutations are lower-case characters.
†Numerical position on the genome of CPV-d, GenBank Accession no. M38245.
v9985-46, v9985-NS, v9985-VP, v9985-G300V, v9985-T389N, v9985-V562L, v9985-N93K/G300V, v9985-N93K/V562L, v9985-G300V/T389N, v9985-G300V/V562L or v9985-T389N/V562L. Dogs were inoculated with the viruses in groups A and B at the highest titres that could be prepared for each group, to determine the virulence of the parental and passaged viruses, i.e. the sizes of the inocula of strains 9985 and 9985-46 were 1×10^5.0 or 1×10^5.5 TCID_{50} ml^{-1} in the CRFK cells, respectively. The animals were also inoculated with the viruses in groups C and D at the highest titres that could be prepared following rescue, to determine virulence. The sizes of the inocula of v9985-NA and v9985-VP were 1×10^5.0 and 1×10^8.0 TCID_{50} ml^{-1} in the CRFK cells, respectively. For the viruses in groups E to N, the size of the inoculum of each rescued virus was normalized to 1×10^5.5 TCID_{50} ml^{-1} in the CRFK cells, except for v9985-N93K/V562L, to 1×10^8.0 TCID_{50} ml^{-1}, which was the highest titre that could be prepared (Table 2). Clinical manifestations of disease in dogs were monitored daily for 14 days and were scored as described by Nakamura et al. [35]. Clinical specimens, including whole blood and rectal swabs, were periodically collected from the dogs and tested for white blood cell count and the presence of infectious virus. Sera were collected from the dogs pre-inoculation and at 7 and 14 days p.i. for measurement of neutralizing antibody titres. Samples intended for viral infectious titre examination were stored at −80 °C prior to analysis. Dogs surviving viral infection were subjected to scheduled euthanasia/necropsy at the completion of the observation time, i.e. 7, 11 or 14 days p.i, as summarized in Table 2. Tissues were collected for histopathological examination.

**Virus shedding**

Virus shedding was assessed by virus titration of rectal swabs. Virus titration was performed by the roller tube method using CRFK cells as described above, except for the use of MEM containing 3000 U ml^{-1} penicillin, 300 µg ml^{-1} streptomycin, 30 µg ml^{-1} kanamycin and 0.6 µg ml^{-1} amphotericin B and the rinsing of cultures once with MEM after the first 24 h. Those cultures that exhibited HA activity were determined positive for CPV, and the viral titres (TCID_{50} ml^{-1}) of the respective samples were calculated using the Reed–Müench method [34].

**Serology**

Serological responses to CPV infections were assessed by serum neutralization (SN) testing with HA as the readout. Briefly, 0.1 ml of serum diluted with culture medium was mixed with 200 TCID_{50} of CPV strain 9985 (0.1 ml) and incubated for 1 h at 37 °C. The mixtures were inoculated into a CRFK cell suspension in roller tubes. After static culture for cell adhesion at 37 °C for 24 h, cultures were incubated on roller drums for 6 days, and inhibition of virus replication was assessed by measuring HA activity of pig erythrocytes with culture supernatant at 4 °C. Those tubes showing no HA activity were determined positive for SN antibodies. SN antibody titres were determined using the Reed–Müench method [34].

**Histopathology**

Intestinal tissues were collected from dogs that had been inoculated with the modified CPVs after euthanasia, either when the dogs met the humane criteria for a study endpoint owing to severe CPV infection or at the end of the observation period at 7 days p.i. Tissues from the jejunum, ileum and colon were formalin fixed, embedded in paraffin, sectioned and stained with haematoxylin–eosin following standard histological procedures. The tissue slides were examined for microscopic lesions, and each lesion was scored from 0 to 3 (normal, 0; mild, 1; moderate, 2; severe, 3). The scoring was based on necrosis of the epithelial cells in the jejunum, ileum and colon; crypt degeneration in the jejunum, ileum and colon; and lymphocyte necrosis in the colon (Fig. S1). Total intestinal histopathological scores for each dog were calculated as a sum of individual lesion scores.

**Statistical analysis**

Two-sample t-tests were employed to evaluate differences between groups comprising different numbers of individuals. Calculations were done using R version 3.1.2 (R Development Core Team, 2014). P values<0.05 were considered significant.

**Funding information**

This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

**Acknowledgements**

We thank Chihiro Kashimoto and Mai Kawamata of Kyoto Biken Laboratories, Inc., for collecting the data used in this study.

**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**Ethical statement**

All experimental procedures that involved animals were conducted in compliance with the regulations and guidelines on animal ethics of Kyoto Biken Laboratories, Inc., which were established by following the Guidelines for Proper Conduct of Animal Experiments as defined by the Science Council of Japan, 1 June 2006, and in compliance with the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Agriculture, Forestry and Fisheries, Japan. 1 June 2006, with prior approval from the Animal Care and Use Committee of Kyoto Biken Laboratories, Inc., which was established and operated in compliance with the above-mentioned guidelines.

**References**


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